

AWARD NUMBER: W81XWH-12-1-0331

TITLE: Apoptosis Induction by Targeting Interferon Gamma Receptor 2 (IFNgammaR2) in Prostate Cancer:
Ligand (IFNgamma)-Independent Novel Function of IFNgammaR2 as a Bax Inhibitor

PRINCIPAL INVESTIGATOR: Shigemi Matsuyama

CONTRACTING ORGANIZATION: Case Western Reserve University
Cleveland, OH 44106-4979

REPORT DATE: October 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188		
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 2016		2. REPORT TYPE Final report		3. DATES COVERED 1 Aug 2012 - 31 Jul 2016	
4. TITLE AND SUBTITLE Apoptosis Induction by Targeting Interferon Gamma Receptor 2 (IFNgammaR2) in Prostate Cancer: Ligand (IFNgamma)-Independent Novel Function of IFNgammaR2 as a Bax Inhibitor			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER W81XWH-12-1-0331		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Shigemi Matsuyama E-Mail: shigemi.matsuyama@case.edu			5d. PROJECT NUMBER		
			5e. TASK NUMBER Task 1		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Case Western Reserve University, School of Medicine, Department of Medicine Hematology/Oncology Division. 10900 Euclid Ave. Cleveland, OH, 44106			8. PERFORMING ORGANIZATION REPORT		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT In our preliminary study, we found that IFN γ R2 has previously unknown function as an inhibitor of Bax. Bax is a key mediator of apoptosis. We found that IFN γ R2 is overexpressed in prostate cancer, and we hypothesize that abnormally high level of IFN γ R2 confers apoptosis resistance of prostate cancer. In this project, we accomplished the following three tasks. Task1: Search for the binding domains of IFN γ R2 and Bax. Our experiments suggest that Bax binding domain may exists in amino acid 301-308 of IFN γ R2, and that IFN γ R2 does not require the N-terminal 53 amino acids of Bax. Task2: Identification of IFN γ R2 expressing PCa: We found that basal cell, but not luminal cell, of prostate tissue of prostate cancer patients expressed high level of IFN γ R2. We also found that IFN γ R2 expression level increases according to the progression of malignancy in cell lines (from androgen-dependent, androgen-independent, and bone metastatic). Furthermore, shRNA-mediated down regulation of IFN γ R2 was able to increase cell death of IFN γ R2 expressing cancer cells both in cell culture and mouse xenograft model. Task3: To determine the mechanism of increased IFN γ R2 expression in PCa. We found that NF κ B inhibitor suppressed IFN γ R2 expression, suggesting that hyper-activation of NF κ B may be one of the mechanisms of IFN γ R2 overexpression in PCa. These results support our hypothesis that IFN γ R2 has an anti-apoptotic activity in PCa and IFN γ R2 is a promising future drug target to improve the efficiency of PCa treatment.					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Introduction

Previously, we identified interferon γ receptor 2 (IFN γ R2) as a Bax suppressor using yeast-based functional screening of Bax inhibiting proteins [1]. Bax is a key mediator of apoptosis which is essential for chemotherapy- and radiation-induced apoptosis of prostate cancer cells[2]. Previously, we reported that IFN γ R2 levels were abnormally elevated in some of prostate cancer cell lines (LNCap and PC3) [1]. Short hairpin (sh) RNA-mediated knockdown of IFN γ R2 was able to increase chemotherapy-induced apoptosis rate significantly in HeLa cells [1] as well as PC3 (a human prostate cancer cell line) cells (unpublished observation, manuscript in preparation), suggesting that IFN γ R2 is a chemo-resistant factor in prostate cancer cells. Although IFN γ R2 was previously known as a receptor of IFN γ which is an anti-tumorigenic cytokine, our preliminary data suggest that IFN γ R2 expresses its anti-apoptosis (anti-Bax) activity independent from IFN γ and IFN γ signaling[1]. Importantly, we found that IFN γ R2 is expressed in mitochondrial membranes and endoplasmic reticulum (ER) membranes, but not on the plasma membranes of prostate cancer cells (unpublished observation, manuscript in preparation). Since we found that IFN γ R2 can directly interact with Bax at least *in vitro* [1], we hypothesize that IFN γ R2 confer apoptosis resistance of prostate cancer by directly binding and inhibiting Bax in intracellular membrane such as endoplasmic reticulum (ER) and mitochondria (Fig.1).

In this project, the following Tasks were accomplished and the detail experimental data are explained in **Results and Discussion** section.

Task 1: To determine the mechanism of Bax inhibition by IFN γ R2 (to identify binding domains of Bax and IFN γ R2), and to develop anti-IFN γ R2 peptide that enhances Bax activation. (Months 1-24)

Task 2: To identify the subtype of prostate cancer that can be effectively treated by IFN γ R2-targeting technologies (Months 13-36)

Task 3: Determination of the mechanism of abnormal expression of IFN γ R2 in prostate cancer (Months 13-36)

Results and Discussion

Task 1a: Search for IFN γ R2 binding domain of Bax

Accomplished experiments 1

We made a series of plasmids that express Bax mutant proteins lacking possible IFN γ R2 binding domains. Bax mutant cDNAs were sub-cloned into Flag-tagged and GFP-tagged protein expression plasmids.

Strategy of making Bax mutants:

Human Bax protein has 198 amino acids. There are 9 alpha helices. The first N-terminal 53 amino acids before the 1st alpha helix is known to function as an auto-inhibitory domain. The 1st alpha helix is also known as BH3 domain that is essential to bind other Bcl-2 family proteins (including Bax itself). Alpha helices 5 and 6 creates channels in the membrane that are required for pore-forming activity of Bax, and this activity is implicated to be essential for cytochrome c release from mitochondria. Alpha helix 9 is a trans-membrane domain that is essential for mitochondrial membrane localization of Bax, and this domain is also essential for apoptosis induction. Each of these domains essential for Bax-mediated apoptosis was deleted, and fused with epitope tag (Flag- or GFP-tag) so that we can detect

expression of these Bax mutants by antibodies detecting epitope tags. Expression of these proteins in HEK293T cells have been confirmed (we will confirm the expression in PC3 soon).

List of newly made plasmid:

*pCMV2B vector express Flag-tagged protein. pEGFP vector express EGFP-tagged protein. Bax full length cDNA has been already subcloned into these vectors.

pCMV2B-Bax Δ N (N-terminal 53 amino acid was deleted)

pCMV2B-Bax $\Delta\alpha$ 1 (α -helix 1 is deleted)

pCMV2B-Bax $\Delta\alpha$ 5-6 (α 5 and α 6 are deleted)

pCMV2B-Bax $\Delta\alpha$ 9 (α 9 is deleted)

pEGFP-Bax Δ N

pEGFP-Bax $\Delta\alpha$ 1

pEGFP-Bax $\Delta\alpha$ 5-6

pEGFP-Bax $\Delta\alpha$ 9

As explained from the next section, these plasmids were used for the search of IFN γ R2 binding domain of Bax.

Accomplished experiments 2

We found that the first 53 amino acid of Bax is not required for IFN γ R2 to inhibit Bax.

The first N-terminal 53 amino acids is known to be auto-inhibitory domain that keeps Bax in the cytosol[3]. If this domain is cleaved, Bax is known to be hyperactive to induce apoptosis. The mechanism by which this N-terminal domain suppresses activation of Bax is not very well known.

We speculated that IFN γ R2 binds to this N-terminal domain to stabilize inactive status of Bax. If it is the case, IFN γ R2 will not be able to suppress apoptosis induced by Bax Δ N (1-53 amino acids are deleted). To examine this speculation, we performed co-transfection of GFP- Bax Δ N and Flag-tagged IFN γ R2 in HEK293 cells (Fig.2).

The conclusion is that IFN γ R2 (both full length and C-terminal 41 amino acids) was able to suppress Bax Δ N –induced apoptosis. This result suggests that IFN γ R2 interacts with unidentified domain in the region of amino acids 54-198 (next section explains the strategy to narrow down the binding domain within this region).

Accomplished experiments 3

Co-immunoprecipitation (co-ip) binding experiments were performed to narrow down IFN γ R2 binding site in Bax protein, however, we were not able to obtain clear conclusion due to the difficulties of the detection of IFN γ R2 in Western blot of co-ip samples (IgG band disturbed the detection) :

Using GFP-Bax plasmids prepared in section 1, we performed co-immunoprecipitation (co-ip) experiments to identify essential domain for the binding of IFN γ R2. IFN γ R2 was expressed as Flag-tagged protein so that we can detect co-immunoprecipitated IFN γ R2 by Flag-antibody. However, we faced a problem. Molecular Weight (MW) of Flag-IFN γ R2 is approximately 52 kDa, and it is very close to 50 kDa IgG Heavy Chain. Unfortunately the 2nd antibody used to detect Flag-tagged IFN γ R2 cross reacted with IgG of anti-GFP antibody that was used to pull down GFP-Bax-Flag-IFN γ R2 complex, and IgG signal interfered the signals from 52 kDa IFN γ R2. In general, to avoid this type of the problem, antibodies from different animal (e.g. the combination of rabbit IgG and mouse IgG) are

used for co-ip and Western blot detection. In our experiment, we used rabbit IgG for GFP antibody and mouse IgG for Flag-IFN γ R2 detection, and the 2nd antibody to detect Flag-IFN γ R2 was anti-mouse IgG, but not anti-rabbit IgG. But, still, there was a cross reactivity. To solve this problem, we made IFN γ R2 antibody conjugated with HRP so that we can detect IFN γ R2 directly without 2nd antibody. Theoretically, we will be able to detect Flag-IFN γ R2 signal without the interference from IgG used for co-ip. Unfortunately, even after several months of attempts to improve the method (antibody concentration, incubation time, for example), we were not able to obtain convincing results even with our homemade IFN γ R2 antibody conjugated with HRP. At present, we think that traditional binding experiment may not be useful to identify precise binding amino acid sequence in Bax. In addition to peptide-based inhibition of IFN γ R2 activity, other strategies targeting IFN γ R2 such as shRNA-mediated down regulation of IFN γ R2 (as explained in Task 2 and 3) may be more promising strategy to develop IFN γ R2-targeting therapy.

Task 1b: Search for Bax binding domain of IFN γ R2

Accomplished experiments 4

Bax binding domain of IFN γ R2 is located in amino acids 296-337 (41 amino acids), and the minimum essential domain may exist in amino acids 301-308.

IFN γ R2 has 337 amino acids. In our preliminary study shown in our proposal, we found that amino acids 296-337 of IFN γ R2 (IFN γ R2 (296-337)) are sufficient to bind Bax and inhibit Bax-induced apoptosis. In addition, we observed that expression of GFP-tagged IFN γ R2 (296-337), but not IFN γ R2 (1-295) suppressed Bax-overexpression-induced apoptosis [1]. These results suggest that Bax binding domain of IFN γ R2 is located in 296-337 (41 amino acids).

Among 41 amino acids in IFN γ R2 (296-337), there is an amino acid sequence of PILEADK (amino acids 301-308) which has a similarity to the Bax binding domain of Ku70 (Ku70 is an Bax inhibiting protein) that was discovered by PI's group[4-7]. Ku70's Bax binding domain is (V)PTLKEA. We examined whether this PILEADK contains Bax inhibiting activity by examining its effects on Bax-mediated cell death (Fig.3), and we observed that penta-peptides (PILEA and EALDK) designed from this sequence has a weak apoptosis inhibition activity. Ku70 derived Bax inhibiting peptide (VPTLK) was used as a positive control for Bax-mediated cell death inhibition. KLPVM is a negative control penta-peptide that does not have Bax inhibiting activity. In the experiment shown in Fig.3, we used Etoposide (10 μ M 16hs and 24 hrs treatment)-induced apoptosis in HeLa cells that is known to be Bax-dependent cell death. All the peptides used in this experiments were tagged with fluorescent tag (Fam) so that peptide entry into the cells can be monitored and confirmed. All the peptides tagged with Fam were able to enter the cell in this experiment. Although further thorough study is clearly necessary to draw conclusion, these results suggest that Bax binding domain in IFN γ R2 may exist in amino acids 301-308 of IFN γ R2.

Task 2: To identify the subtype of prostate cancer that can be effectively treated by IFN γ R2-targeting

Accomplished experiments 5

Immunohistochemistry of Tissue Microarray were performed.

We performed two different approaches to investigate IFN γ R2 expression patterns in prostate cancer. One is to examine the correlation between IFN γ R2 expression and clinical outcome using patient specimen (please see Accomplished experiment 7), and another one is to utilize commercially available tissue microarray of human prostate cancer. the second strategy and examined 11 prostate cancer patient samples together with 8 normal prostate specimen as controls. The summary is presented in **Fig.4**.

We found that majority of progressed prostate cancer specimen showed elevated expression of IFN γ R2, and these cells also express Bax. Some of advanced prostate cancer (such as No. 1-4, 9, 10 and 11 Fig.4) expresses high levels of IFN γ 2 as well as Bax, but no detectable Bcl-2. In this type, prostate cancer cells may suppress Bax-induced apoptosis mainly by depending on IFN γ 2, and thus IFN γ R2 targeting therapy is expected to work well. In the case of prostate cancer expressing both IFN γ R2 and Bcl2, combinational treatment of IFN γ R2 inhibitor and Bcl2 inhibitor (such as ABT-263 derivatives) may be effective.

Accomplished experiments 6

IFN γ R2 expression patterns in prostate cancer were investigated by analyzing human patient database.

Using Oncomine database (publically available gene expression profile database of human cancer patients), we found that IFN γ R2 expression levels of prostate cancer are significantly higher than normal prostate as we expected. To determine the significance of IFN γ R2 elevation in apoptosis-resistance of prostate cancer, we also checked the expression levels of other well-known apoptosis inhibiting proteins such as Bcl-2, Bcl-XL, and Mcl-1. Results are presented in **Fig.5**. Very interestingly, only IFN γ R2 (3.69 times increase, $p < 0.000115$) and Mcl-1 (3.24 times increase, $p < 0.000319$) showed significant increased expression in prostate cancer in comparison with normal prostate, but not Bcl-2 and Bcl-XL (BCLL1 in the figure). Expression of apoptosis inducer such as Bax and Bak did not show remarkable changes. These results suggest that IFN γ R2 and Mcl1, but not Bcl-2 and BclXL, are the ideal targets to induce Bax/Bak-mediated apoptosis in prostate cancer. This information also suggests that Mcl1 inhibition may be also necessary to induce prostate cancer cell death when IFN γ R2 inhibition is used to enhance Bax-induced apoptosis.

Accomplished experiments 7

Significant IFN γ R2 expression was detected in prostate basal cells (hyperplasia area) of patients who experienced recurrence after prostatectomy and radiation therapy.

In Figs. 6-7, representative results are shown. Fig.6 (A and B) show IFN γ R2 expressing cells were clearly detected in prostates of patients with recurrence (in all 4 patients examined), but IFN γ R2 expressing cells were NOT always found in prostates of patients who did not have recurrence (2 out of 3 patients did not have IFN γ R2 positive cells) (Fig. 6C). Interestingly, IFN γ R2 was detected in basal cells, but not in luminal cells. Furthermore, IFN γ R2 is not expressed in all basal cells, but only in basal cells that show hyperplasia destructing the tube structure of prostate gland (Fig. 6A) or in basal cells in a few prostate glands that still maintains normal structure (Fig. 6B). We speculate that Fig.6B shows the emergence of tumor-initiating basal cells in particular area, and Fig. 6A shows the example of pre-tumorigenic growth of basal cells. In Fig. 7, other pictures of IFN γ R2 positive prostate cells are displayed in the order of the progression of prostate cancer. Fig.7D shows the example of the destruction of luminal structure by abnormal growth of basal cells that express IFN γ R2. Fig.7E is the results in metastatic prostate cancer section showing that majority of cancer cells express high level of IFN γ R2. Interestingly, IFN γ R2 was not detected in luminal cell type prostate cancer (see Fig. 6C) suggesting that IFN γ R2 expression may decrease when basal cell differentiates into luminal cells. Since IFN γ R2 protects prostate cancer cells from apoptosis, IFN γ R2 (-) luminal cell type cancer cells are likely treatable (i.e. relatively easy to induce apoptosis) cancer and IFN γ R2 (+) basal cells may represent apoptosis-resistant cancer initiating cells or cancer stem cells (Fig. 8).

Accomplished experiments 8

IFN γ R2 expression increases according to the progression of malignancy of prostate cancer cells.

Please see Fig.9 We examined expression levels of IFN γ R2 in widely used prostate cancer cell lines. These cell lines were originated from the same cell line to investigate the molecular mechanism of

progression of prostate cancer, i.e. from androgen-dependent state (LNCaP) to androgen-independent state (C4-2), and to bone metastatic state (C4-2B). Interestingly, IFN γ R2 levels increased according to the progression of malignancy in these prostate cancer cells. It has been known that C4-2B has constitutive active androgen receptor (AR). Therefore, elevated activity of AR may contribute to the increased expression of IFN γ R2.

Since C4-2B expresses high level of IFN γ R2, we speculated that IFN γ R2's role to suppress cell death is significant in this cell line. In fact, IFN γ R2 knock down significantly slowed down the growth of C4-2B and induce spontaneous cell death as seen in Fig. 10. These results suggest that IFN γ R2 has a significant role to suppress apoptosis in aggressive prostate cancer that shows androgen independency and metastatic activity.

Task 3: Determination of the mechanism of abnormal expression of IFN γ R2 in prostate cancer (Months 13-36)

Accomplished experiments 9

Effectiveness of IFN γ R2 inhibition to promote prostate cancer cell death was examined using mouse xenograft experiments.

We prepared two cancer cell lines in which IFN γ R2 was knocked down by shRNA. These cell lines are PC3 (human prostate cancer cell line) and A375 (human melanoma cell line). We examined A375 cell line, since we found that this cell line also expresses high levels of IFN γ R2.

We injected one million cells of cancer cells to each nude mouse, and docetaxel (1mg/kg) and cisplatin (5mg/kg) were treated every week to mice. In PC3 cell line experiments, more than a half of docetaxel-treated mice (2-3 out of 4 mice) were dead within two weeks of treatment, therefore we were not able to obtain reportable result. In the case of cisplatin-treated A375 experiments, we were able to obtain preliminary results to determine the effects of IFN γ R2 knockdown. IFN γ R2 knockdown was able to slow down the growth of tumor (Fig.11 panel B) in comparison with control shRNA expressing cells (Fig.11 panel A). Tumor growth after cisplatin-treatment was also suppressed by shRNA-mediated IFN γ R2 knockdown (Fig.11 panel D) in comparison with control (Fig.11 panel C), though whether cisplatin-induced cell death was "enhanced" is not yet clear, since IFN γ R2 shRNA alone (without cisplatin) showed significant suppression of tumor growth (Fig.11 panel A vs B).

Accomplished experiments 10

NF κ B inhibitor (Parthenolide) suppressed IFN γ R2 expression

To develop technologies targeting IFN γ R2, we proposed to determine the effectiveness of currently available drugs that is predicted to decrease IFN γ R2 expression in prostate cancer. Since previous studies have shown that NF κ B is one of transcription factors that stimulate IFN γ R2 gene expression [8], we proposed to determine the effects of NF κ B inhibitor. Parthenolide is a plant-derived compound which is known to inhibit NF κ B activity[9, 10]. In our preliminary study, we found that parthenolide was able to decrease IFN γ R2 expression in PC3 prostate cancer cells. In this study, we examined another standard prostate cancer cells, LNCaP cell line (Fig.12). We found that IFN γ R2 expression was suppressed by parthenolide (from 5 μ M) within 1 day after the treatment (Fig. 12 shows the result of 1 day treatment). Importantly, Bax expression was not decreased by this treatment, suggesting that parthenolide can stimulate Bax-mediated cell death by decreasing Bax inhibitor, i.e. IFN γ R2.

Methods:

Cell culture and plasmid transfection:

HEK293T cells and PC3 cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and 1% penicillin/streptomycin. For plasmid transfection to examine Bax inhibiting activity and Bax binding activity of IFN γ R2, 2×10^5 cells/6 cm dish and 1×10^6 cells/10 cm dish, respectively, were used. Plasmid transfection were performed by using Superfect (Qiagen). For bax inhibiting activity, 0.5 ug Bax expressing plasmid and 2.0 ug IFN γ R2 expressing plasmids were co-transfected to 6 cm dish culture. For Bax binding experiments (co-immunoprecipitation experiments), 1 ug of Bax expressing plasmids and 4 ug of IFN γ R2 expressing plasmids were transfected to the cells cultured in 10 cm dish. 24 hrs after the transfection, cells were collected for apoptosis analysis or cell lysate preparation for binding experiments as described below.

Generation of Plasmid:

pCMV vector and pEGFP vectors were purchased from Clontech. Bax mutant cDNAs were generated by 2 step PCR methods, and these mutant cDNAs were subcloned into pCMV and pEGFP vectors.

Peptide Synthesis:

Peptides were purchased from Biopeptide (San Diego) by order made. Peptides were purified by HPLC and the purity is more than 99% so that possibly toxic chemicals were removed from the peptide solution. Peptides were dissolved in PBS at the concentration of 100 mM, and 200 uM were added to the cell culture to examine whether the peptide can inhibit Bax-mediated cell death.

Cell Death Assay:

After 24 hrs of plasmid transfection, cells were re-suspended in HBSS (Hanks Buffered Saline Solution) at 4C, and Hoechst 33258 (DNA staining fluorescent dye, purchased from Sigma) were added at 10 ug/ml concentration. Under fluorescent microscope, transfected cells were identified by GFP signal, and percentages of apoptotic cells showing apoptotic nuclear fragmentation or condensation among GFP positive cells were measured. Total 300 cells were counted in each sample.

Binding experiments:

Cells were lysed by lysis buffer (1% NP40 10 mM HEPES and 140 mM NaCl) containing protease inhibitor cocktail (purchased from Sigma). Cell lysates are adjusted to 2.5 ug/ul protein concentration by the lysis buffer. 200 ul of cell lysates were pre-cleaned by shepharose beads, and the cleaned lysates were subjected to co-immunoprecipitation using anti-GFP antibody. After 2 hrs of incubation with GFP antibody, immune-complex were pulled down by Protein A coated Sepharose beads (Pharmacia). After 3 times wash of the beads by lysis buffer, immunocomplex was eluted by 50ul of Lamli buffer for SDS-PAGE. Twenty ul of sample were applied to each lane of SDS-PAGE and Western blot was performed by using anti-flag antibody.

Cell culture and plasmid transfection:

HEK293T cells and PC3 cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and 1% penicillin/streptomycin. For plasmid transfection to examine Bax inhibiting activity and Bax binding activity of IFN γ R2, 2×10^5 cells/6 cm dish and 1×10^6 cells/10 cm dish, respectively, were used. Plasmid transfection were performed by using Superfect (Qiagen). For bax inhibiting activity, 0.5 ug Bax expressing plasmid and 2.0 ug IFN γ R2 expressing plasmids were co-transfected to 6 cm dish culture. For Bax binding experiments (co-immunoprecipitation experiments), 1 ug of Bax expressing plasmids and 4 ug of IFN γ R2 expressing plasmids were transfected to the cells cultured in 10 cm dish. 24 hrs after the transfection, cells were collected for apoptosis analysis or cell lysate preparation for binding experiments as described below.

Generation of Plasmid:

pCMV vector and pEGFP vectors were purchased from Clontech. Bax mutant cDNAs were generated by 2 step PCR methods, and these mutant cDNAs were subcloned into pCMV and pEGFP vectors.

Peptide Synthesis:

Peptides were purchased from Biopeptide (San Diego) by order made. Peptides were purified by HPLC and the purity is more than 99% so that possibly toxic chemicals were removed from the peptide solution. Peptides were dissolved in PBS at the concentration of 100 mM, and 200 uM were added to the cell culture to examine whether the peptide can inhibit Bax-mediated cell death.

Cell Death Assay:

After 24 hrs of plasmid transfection, cells were re-suspended in HBSS (Hanks Buffered Saline Solution) at 4C, and Hoechst 33258 (DNA staining fluorescent dye, purchased from Sigma) were added at 10 ug/ml concentration. Under fluorescent microscope, transfected cells were identified by GFP signal, and percentages of apoptotic cells showing apoptotic nuclear fragmentation or condensation among GFP positive cells were measured. Total 300 cells were counted in each sample.

Binding experiments:

Cells were lysed by lysis buffer (1% NP40 10 mM HEPES and 140 mM NaCl) containing protease inhibitor cocktail (purchased from Sigma). Cell lysates are adjusted to 2.5 ug/ul protein concentration by the lysis buffer. 200 ul of cell lysates were precleaned by shepharose beads, and the cleaned lysates were subjected to co-immunoprecipitation using anti-GFP antibody. After 2 hrs of incubation with GFP antibody, immunocomplex were pulled down by Protein A coated Sepharose beads (Pharmacia). After 3 times wash of the beads by lysis buffer, immunocomplex was eluted by 50ul of Lamli buffer for SDS-PAGE. Twenty ul of sample were applied to each lane of SDS-PAGE and Western blot was performed by using anti-flag antibody.

shRNA-mediated down regulation of IFN γ R2 in C4-2B cell line

IFN γ R2 targeting shRNA was introduced into cells by using lentivirus transfection system (Thermo Fisher Scientific, USA). Control shRNA encodes shRNA against Green Fluorescent Protein (GFP) that does not exist in human cells. Cells successfully transfected by lentivirus were selected by puromycin, and cell lysates were collected to determine IFN γ R2 protein expression.

Immunohistochemistry of human prostate cancer tissue microarray.

Human prostate cancer tissue microarray was purchased from BioMax (Maryland, USA). Immunohistochemistry of IFN γ R2 was performed by the standard methods explained in detail in Abcam website (<http://www.abcam.com/index.html?pageconfig=resource&rid=13046>). Antibodies used in these experiments is: IFN γ R2 (Abcam, #ab77246).

Cell culture and cell lysate preparation for Western blot

LNCaP, C4-2, and C4-2B cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and 1% penicillin/streptomycin. Cell lysates were prepared by solubilizing cells using 1% NP40 containing HEPES buffer. Insoluble fraction was removed by centrifuge separation (14k rpm for 20n min at 4C). For the analysis of protein expression, cell lysates containing 10 ug protein were used. SDS-PAGE was performed by using 4-20% gradient gel, and immuno-detection was performed by ECA Chemical luminescence detection kit (Amersham).

Immunohistochemistry of human prostate cancer tissue microarray.

Human prostate cancer tissue microarray was purchased from BioMax (Maryland, USA). Immunohistochemistry of IFN γ R2, Bax, and Bcl-2 were performed by the standard methods explained in detail in Abcam website (<http://www.abcam.com/index.html?pageconfig=resource&rid=13046>). Antibodies used in these experiments are: IFN γ R2 (Abcam, #ab77246), Bax (BD Pharmingen #554104), and Bcl-2 (BD Pharmingen #514202).

Definition of tumor grade

The Grade 1-3 in Pathology Diagnosis is equivalent to well-differentiated, moderately-differentiated or poorly differentiated, respectively, under microscope.

Grade 1 or well-differentiated: Cells appear normal and are not growing rapidly.

Grade2 or moderately-differentiated: Cells appear slightly different than normal.

Grade3 or poorly differentiated: Cells appear abnormal and tend to grow and spread more aggressively.

Grade 4 or undifferentiated: *(for certain tumors), features are not significantly distinguishing to make it look any different from undifferentiated cancers which occur in other organs.

TNM grading:

T - Primary tumor

Tx - Primary tumor cannot be assessed

T0 - No evidence of primary tumor

Tis - Carcinoma in situ; intraepithelial or invasion of lamina propria

T1 - Tumor invades submucosa

T2 - Tumor invades muscularis propria

T3 - Tumor invades through muscularis propria into subserosa or into non-peritonealized pericolic or perirectal tissues

T4 - Tumor directly invades other organs or structures and/or perforate visceral peritoneum

N - Regional lymph nodes

Nx - Regional lymph nodes cannot be assessed

N0 - No Regional lymph node metastasis

N1 - Metastasis in 1 to 3 regional lymph nodes

N2 - Metastasis in 4 or more regional lymph nodes

M - Distant Metastasis

Mx - Distant metastasis cannot be assessed

M0 - No distant metastasis

M1 - Distant metastasis

Cell culture and cell lysate preparation for Western blot

PC3 and LNCaP cells were obtained from ATCC, and these cells were cultured in DMEM containing 10%FCS and 1% penicillin/streptomycin. To determine the effects of parthenolide, cells were cultured in the presence of various concentration of parthenolide (5, 10, or 20 ug/ml) for 1 day. Cell lysates were prepared by solubilizing cell pellets using 1% NP40 containing HEPES buffer. Insoluble fraction was removed by centrifuge separation (14k rpm for 20n min at 4C). For the analysis of protein expression, cell lysates containing 10 ug protein were used. SDS-PAGE was performed by using 4-20% gradient gel, and immuno-detection was performed by ECA Chemical luminescence detection kit (Amersham).

Mouse xenograft experiments

One million cells of cancer cells (PC3 and A375) were subcutaneously injected to nude mice. One week later, docetaxel (1 mg/kg) or cisplatin (5 mg/kg) were administered (i.p. injection) once a week, for 4-5 weeks. When tumor size reaches 10% of mouse body, experiments were stopped, and mice were euthanized for tumor size analysis.

Key Research Accomplishment

1. Plasmids that express GFP- and Flag-tagged Bax mutant proteins were prepared.
2. We found that The N-terminus of Bax is not essential for IFN γ R2 to inhibit Bax-induced apoptosis.

3. We confirmed that Bax binding domain of IFN γ R2 is localized in the C-terminal 41 amino acids of IFN γ R2 (IFN γ R2 (263-337) was able to inhibit Bax-induced apoptosis).
4. Bax binding domain of IFN γ R2 is located in amino acids 296-337 (41 amino acids), and the minimum essential domain may exist in amino acids 301-308.
5. Significant IFN γ R2 expression was detected in prostate basal cells (hyperplasia area) of patients who experienced recurrence after prostatectomy and radiation therapy.
6. We found that IFN γ R2 expression increases according to the progression of malignancy of prostate cancer cells.
7. We found that shRNA-mediated IFN γ R2 inhibition was able to increase cancer cell death in mouse xenograft experiment.
8. NF κ B inhibitor (Parthenolide) suppressed IFN γ R2 expression

Reportable Outcome

We presented this study in the international meeting of interferon held in Melbourne, Australia, in November 2014. Actually, PI was invited as a selected speaker in this meeting. All the key research accomplishments are reportable results for the future publication. We are preparing an article to be submitted in 2017 that will report the effects of shRNA in C4-2B prostate cancer cells, mouse xenograft experiment result, IFN γ R2's subcellular localization study, and the striking IFN γ R2 staining pattern in prostate cancer tissue (only basal cell in particular area become IFN γ R2 positive).

References:

1. Gomez, J.A., et al., *The C-terminus of interferon gamma receptor beta chain (IFN γ ammaR2) has antiapoptotic activity as a Bax inhibitor*. Cancer Biol Ther, 2009. **8**(18): p. 1771-86.
2. Korsmeyer, S.J., *BCL-2 gene family and the regulation of programmed cell death*. Cancer Res, 1999. **59**(7 Suppl): p. 1693s-1700s.
3. Suzuki, M., R.J. Youle, and N. Tjandra, *Structure of Bax: coregulation of dimer formation and intracellular localization*. Cell, 2000. **103**(4): p. 645-54.
4. Yoshida, T., et al., *Bax-inhibiting peptide derived from mouse and rat Ku70*. Biochem Biophys Res Commun, 2004. **321**(4): p. 961-6.
5. Gomez, J.A., et al., *Bax-inhibiting peptides derived from Ku70 and cell-penetrating pentapeptides*. Biochem Soc Trans, 2007. **35**(Pt 4): p. 797-801.
6. Gomez, J.A., et al., *Cell-Penetrating Penta-Peptides (CPP5s): Measurement of Cell Entry and Protein-Transduction Activity*. Pharmaceuticals (Basel), 2010. **3**(12): p. 3594-3613.
7. Gomez, J. and S. Matsuyama, *Cell-penetrating penta-peptides and Bax-inhibiting peptides: protocol for their application*. Methods Mol Biol, 2011. **683**: p. 465-71.
8. Ebensperger, C., et al., *Genomic organization and promoter analysis of the gene IFN γ r2 encoding the second chain of the mouse interferon-gamma receptor*. Scand J Immunol, 1996. **44**(6): p. 599-606.
9. Shanmugam, R., et al., *A water-soluble parthenolide analogue suppresses in vivo prostate cancer growth by targeting NF κ B and generating reactive oxygen species*. Prostate, 2011. **70**(10): p. 1074-86.
10. Sun, Y., et al., *The radiosensitization effect of parthenolide in prostate cancer cells is mediated by nuclear factor-kappaB inhibition and enhanced by the presence of PTEN*. Mol Cancer Ther, 2007. **6**(9): p. 2477-86.

Fig.2. The N-terminal 53 amino acids of Bax is not required for Bax inhibition by IFN γ R2. Bax or Bax without 1-53 amino acids (Bax Δ N) were expressed as GFP-fusion protein together with Flag-tagged IFN γ R2 (full length) or -IFN γ R2 (263-337). GFP vector or Flag-tagged control protein (firefly luciferase) were used as a negative control. IFN γ R2 and IFN γ R2 (263-337) were able to inhibit apoptosis induced by both Bax and Bax Δ N, suggesting that the first 53 amino acids of Bax is not the target of IFN γ R2 to inhibit Bax-induced apoptosis.

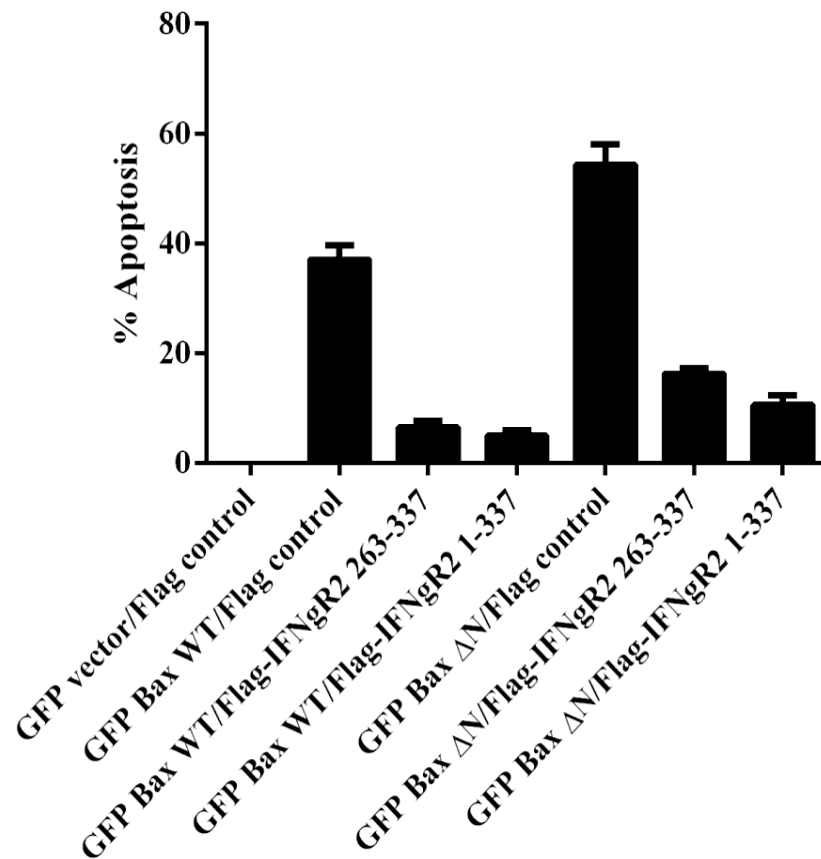


Fig.3. Peptides designed from IFN γ R2 may have an anti-apoptotic activity.

HeLa cells were treated with etoposide (10 μ M) (Eto) in the presence or absence of Fam (FITC fluorescence tag)-labeled peptides (200 μ M). Cells were incubated for 16 or 24 hrs, and apoptosis was detected by Hoechst dye nuclear staining. Four types of peptides were tested. VPTLK: Bax inhibiting peptide (Bax inhibition has been confirmed previously) designed from Ku70 protein. KLPVM: Negative control peptide that does not have an activity to inhibit Bax. PILEA: Peptide designed from amino acids 301-305 of IFN γ R2. EALDK: Peptide designed from amino acids 304-308 of IFN γ R2. Peptides dissolved in DMSO were added to the culture medium so that the final concentration of DMSO will become 0.1 %. For negative control experiment, DMSO (0.1%) was used. Bck: Background cell death in complete negative control group (no DMSO, no drugs, no peptide).

A: Effects of peptides on etoposide-induced cell death are shown. B and C: Confirmation of the entry of Fam-tagged peptide (PILEA is shown as an example) in the cell (B: 10X objective lenses, C: 40X objective lenses)

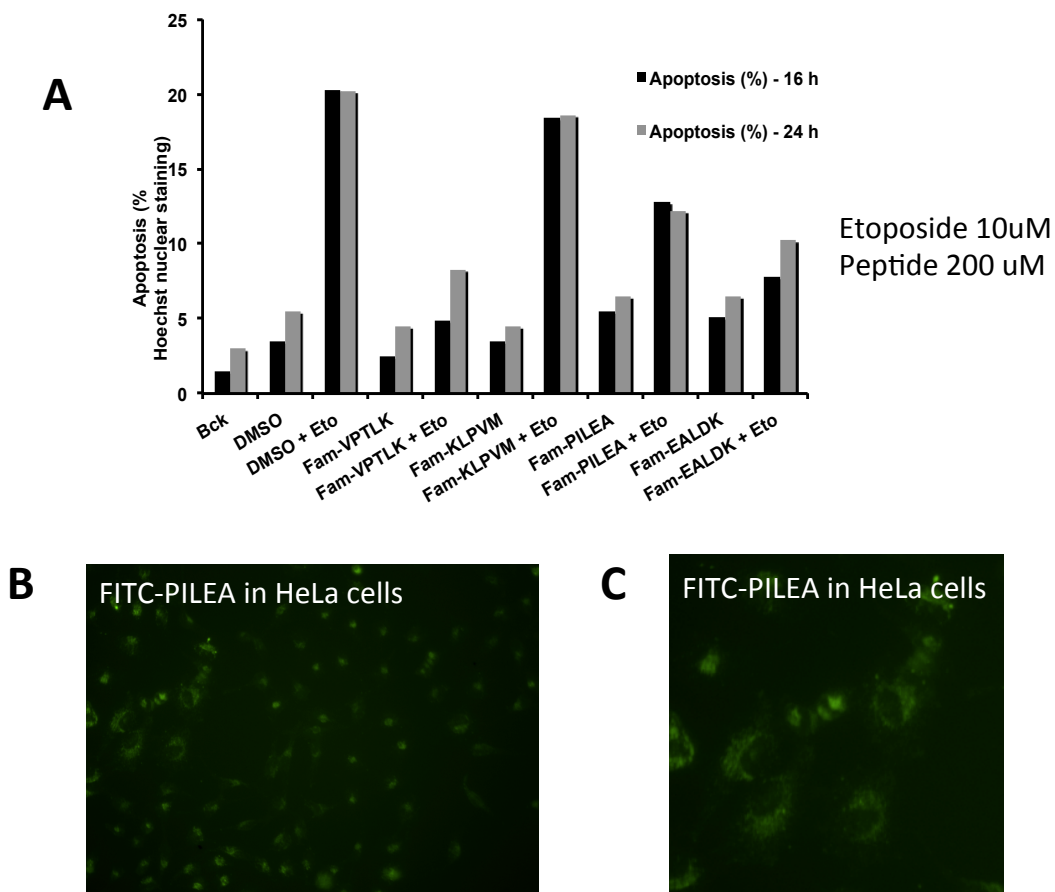
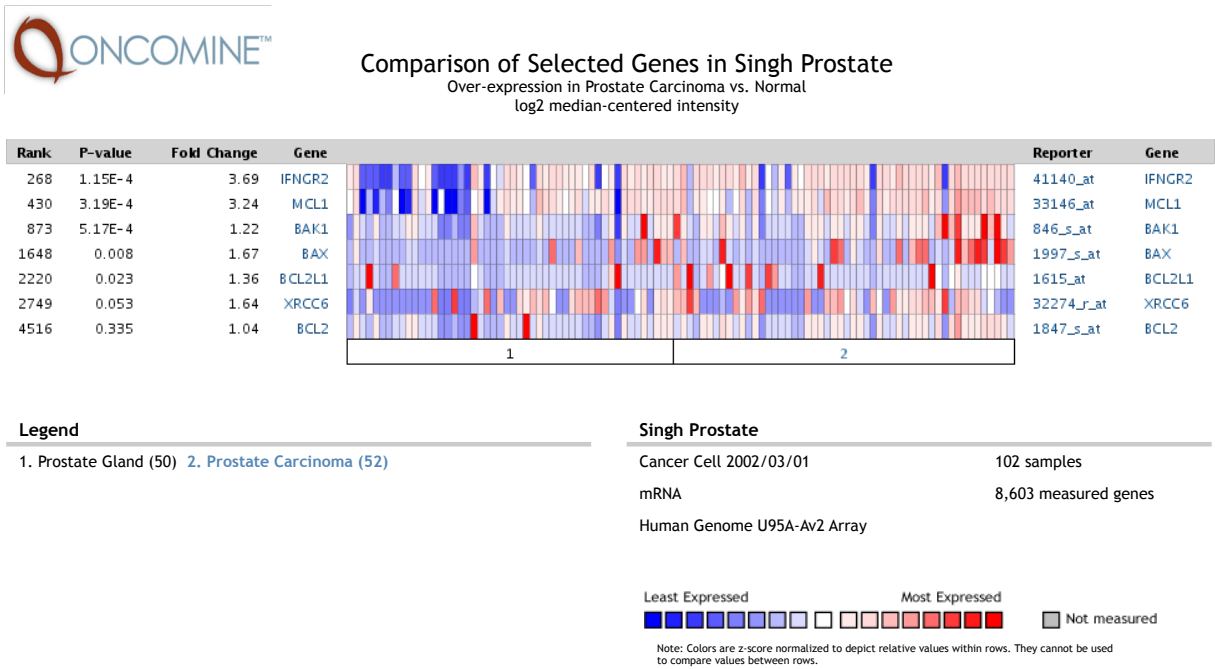


Fig.4 Increased expression of INFgR2 was observed in human prostate cancer tissue microarray. (Please see the definition of cancer progression grade (Grade) and metastasis activity (TNM) in the last page of the proposal)

No.	Position	Age	Grade	TNM	INFgR2	Bax	Bcl-2
1	A1	69	2	T2N0M0	++	+	-
2	A2	69	2	T2N0M0	±	+	-
3	A3	76	2-3	T3N1M1b	± ~ ++	+	+
4	A4	76	2-3	T3N1M1b	++	+	±
5	A5	69	2	T2N0M0	+	-	-
6	A6	69	2	T2N0M0	+	-	-
7	A7	76	2-3	T3N1M1b	±and++	-	-
8	A8	76	2-3	T3N1M1b	+	±	-and+
9	B1	72	3-4	T3N0M0	+	+	+(N)
10	B2	72	3-4	T3N0M0	+ ~ ±	+	+(N)
11	B3	60	4	T2N0M0	- ~ ±	+	+and-
12	B4	60	4	T2N0M0	±	±	+and-
13	B7	60	4	T2N0M0	± ~ +	-	+
14	B8	60	4	T2N0M0	±	±	-
15	B5	72	hyperplasia	T3N0M0	± ~ ++	-and+	±
16	B6	72	hyperplasia	T3N0M0	++	-and+	+
17	C1	43	Normal	-	-	-	-
18	C2	43	Normal	-	-	-	-
19	C3	28	Normal	-	-	±	±
20	C4	28	Normal	-	±	±	±
21	C5	43	Normal	-	-	-	-
22	C6	43	Normal	-	-	-	-
23	C7	28	Normal	-	- ~ ±	-	±
24	C8	28	Normal	-	- ~ ±	±	-

Fig.5 IFNgR2 expression is higher in prostate cancer than that in normal prostate



Copyright© Oncomine, 2008 Images from Oncomine™ may be used in publications with proper citation. The citation for Oncomine is as follows: Oncomine™ (Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization. For further information, refer to the terms described in the license agreement.

Oncomine Source: [https://www.oncomine.com/resource/main.html#a:1947;cs:sizeSmallLarge;cv:detail;d:258;dso:geneOverex;dt:predefinedClass;ec:\[2\];epv:150001.151078;et:over;f:2506691;g:3460;p:200001343;pg:1;pvf:3483,5673,5699,5742,5744,15271,27047,39565,150004;scr:datasets;ss:analysis;th:g100.0,p0.050,fc2.0;v:10](https://www.oncomine.com/resource/main.html#a:1947;cs:sizeSmallLarge;cv:detail;d:258;dso:geneOverex;dt:predefinedClass;ec:[2];epv:150001.151078;et:over;f:2506691;g:3460;p:200001343;pg:1;pvf:3483,5673,5699,5742,5744,15271,27047,39565,150004;scr:datasets;ss:analysis;th:g100.0,p0.050,fc2.0;v:10)

Fig.6. Immunohistochemistry of IFN γ R2 in human prostate. A and B: IFN γ R2 staining (brown cells, stained by HRP (Horse Radish Peroxidase)-DAB (Diaminobenzidine)) is detected in basal cells, but not in luminal cells or other cell types in prostate. C: IFN γ R2 was not detected in prostate cancer area. Note: A and B are results of prostates from patients with recurrence. C is from a patient with no recurrence.

Figure 6

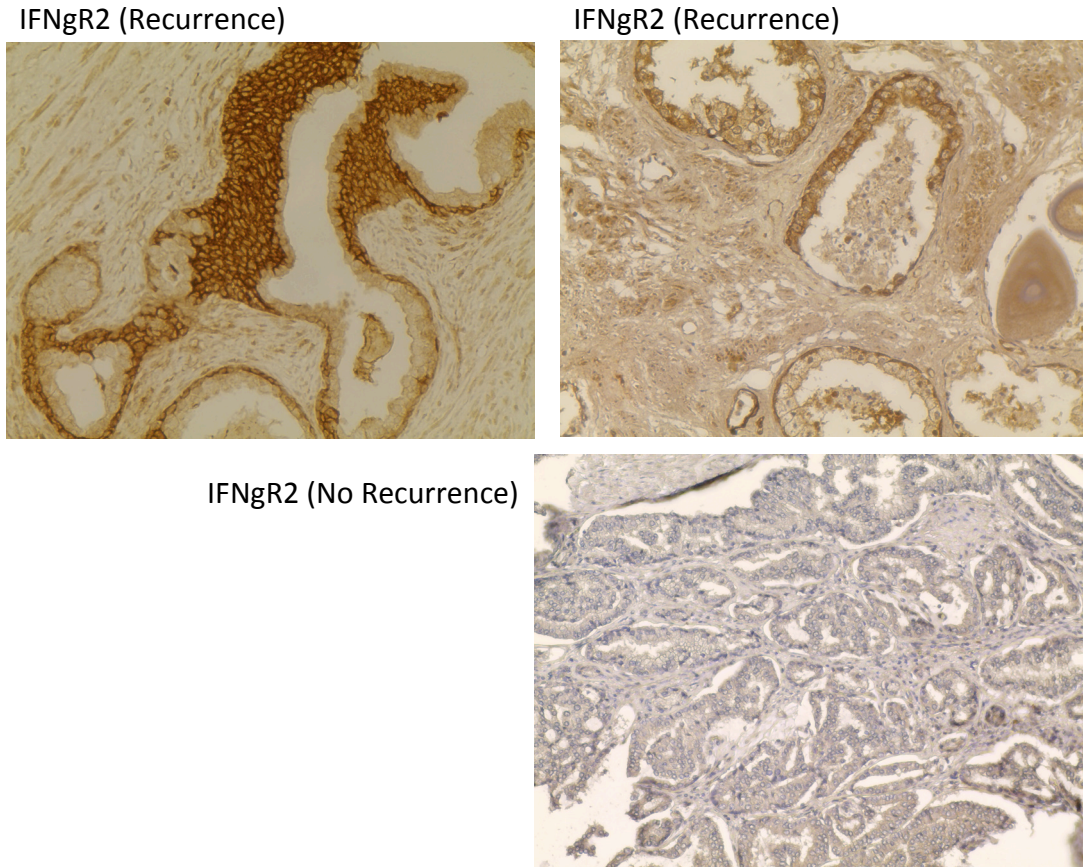


Fig.7. Summary of immunohistochemistry experiments of IFN γ R2 in prostates of patients with recurrence. From A to D, an emergence and growth of abnormal basal cells expressing IFN γ R2 are shown. E shows IFN γ R2 positive prostate cancer cells that showed bone metastasis.

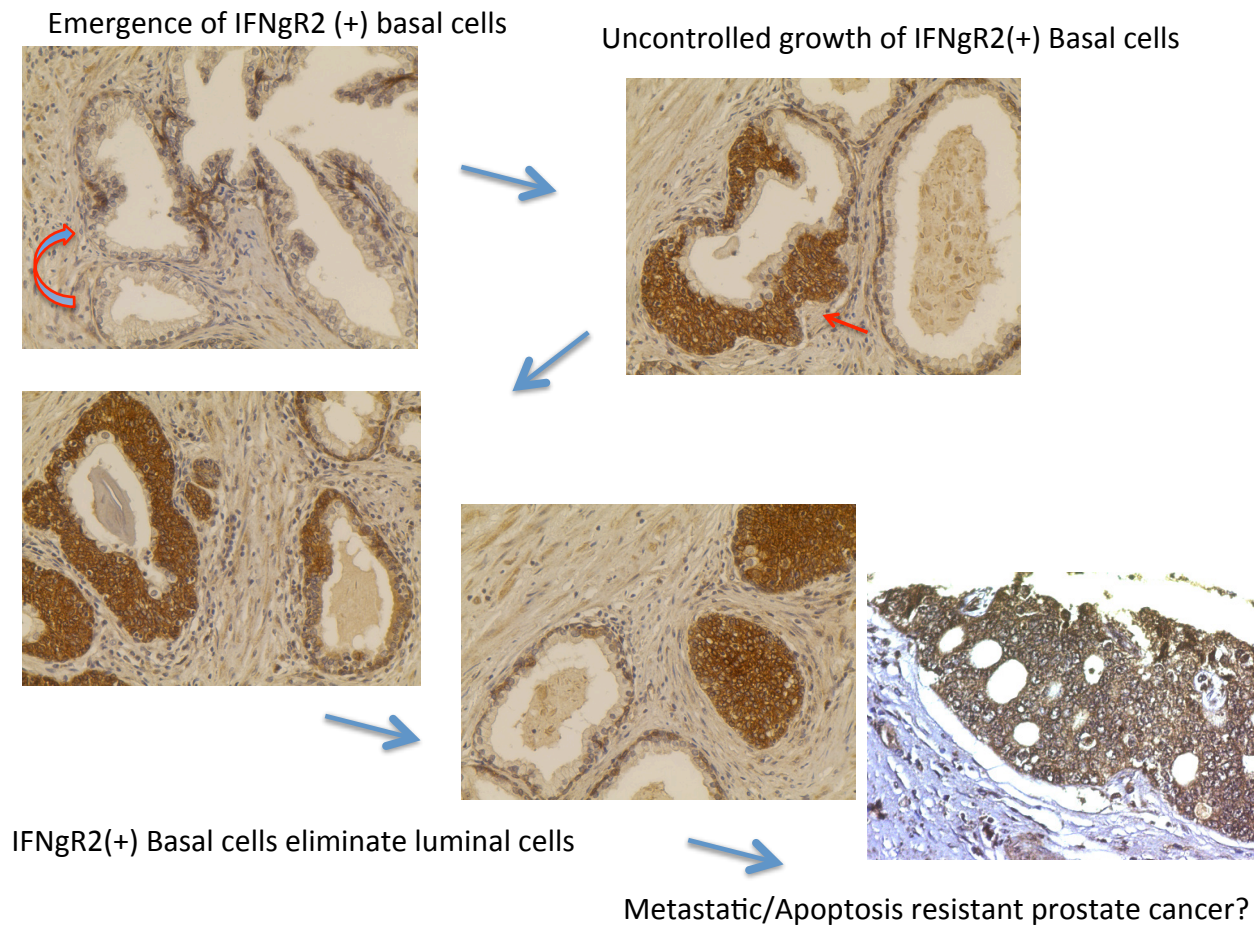
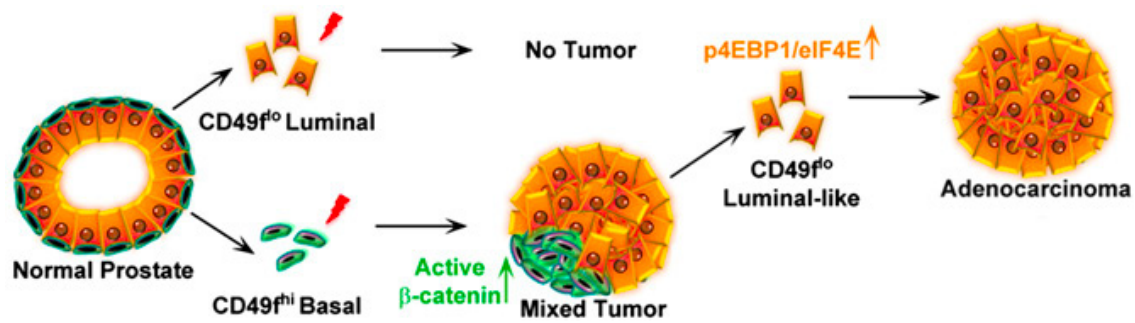


Fig.8

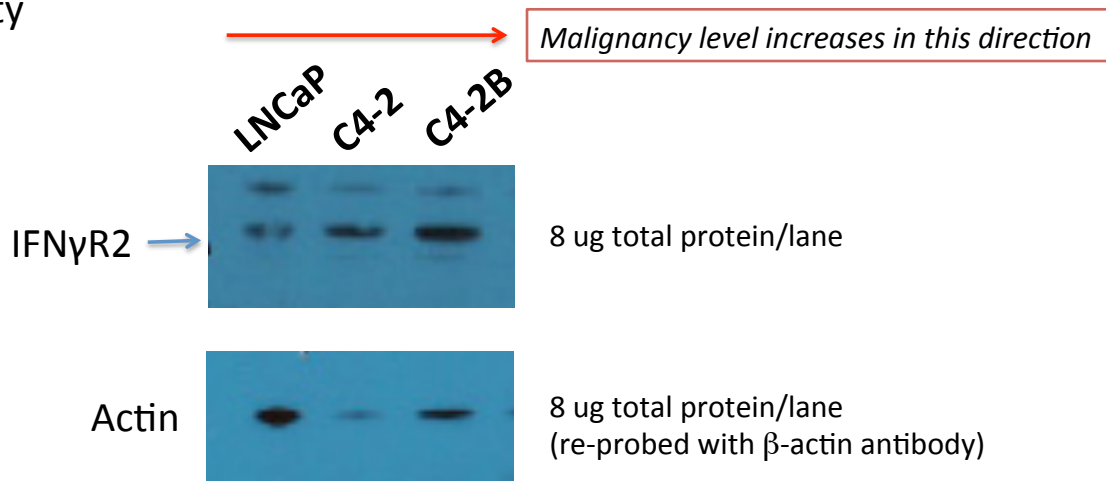
IFNgR2 is expressed in a subgroup of basal cells showing hyperplasia



Basal cell acquires IFNgR2 overexpression, and become tumor initiating cells??

Model of human prostate cancer initiation and propagation by distinct phenotypic cell populations (Modified from *Stoyanova et al. PNAS 2013*)

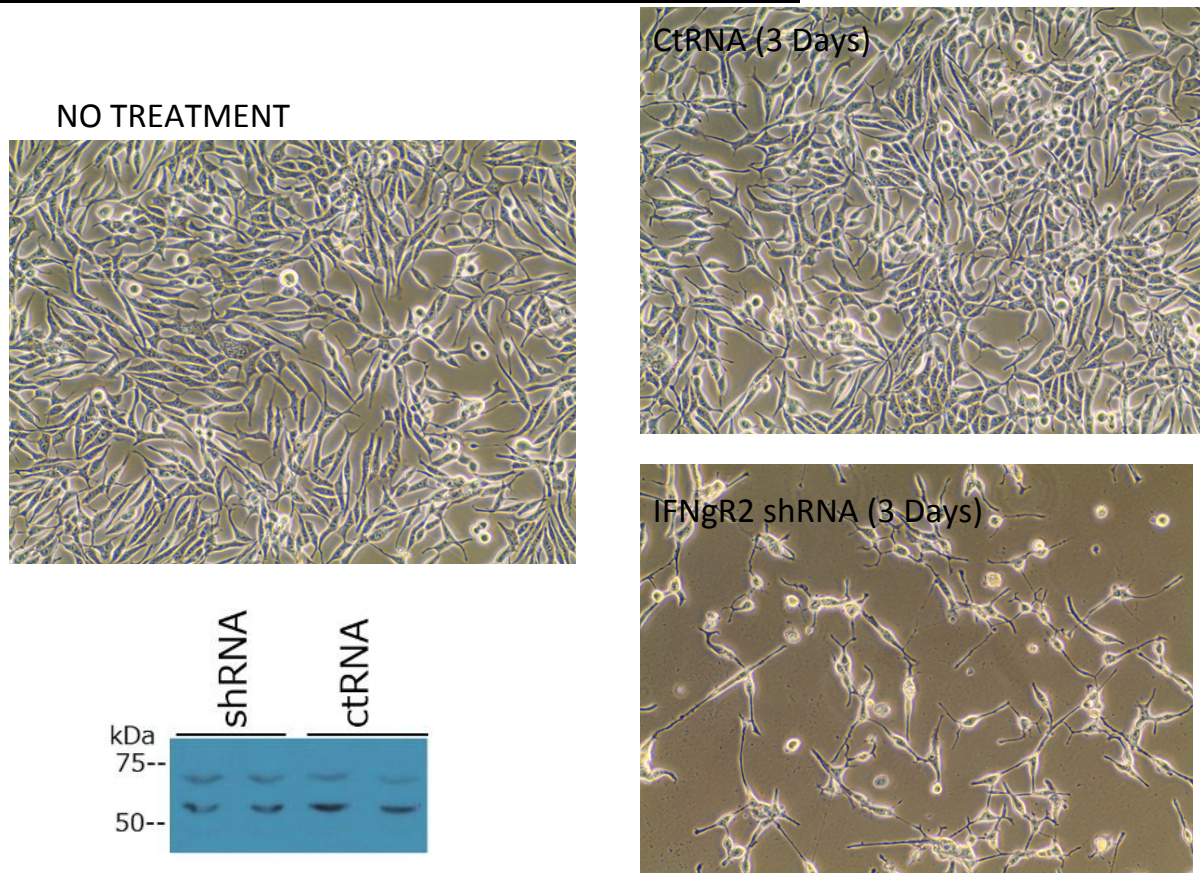
Fig.9: IFN γ R2 expression levels become higher when prostate cancer cell line (LNCaP) acquire androgen-independency and bone metastasis capability



*LNCaP has a Jak1 inactivating mutation, and thus IFN γ does not activate canonical IFN γ signaling pathway.

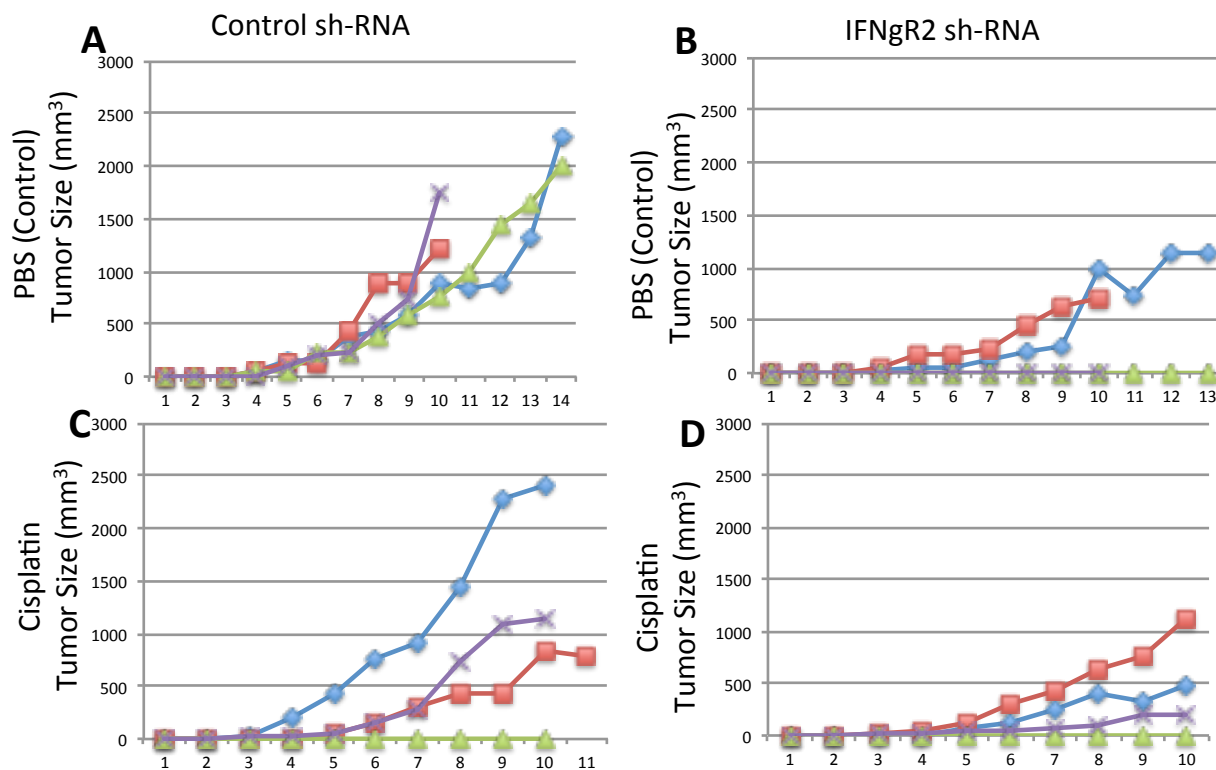
C4-2 and C4-2B cell lines were generated from LNCaP cell line, which is an androgen-dependent cell line. C4-2 is androgen-independent, and C4-2B is androgen-independent and bone metastatic cell lines. These 3 cell lines are used to study the mechanism of prostate cancer progression.

Fig.10 IFN γ R2 shRNA suppressed C4-2B growth



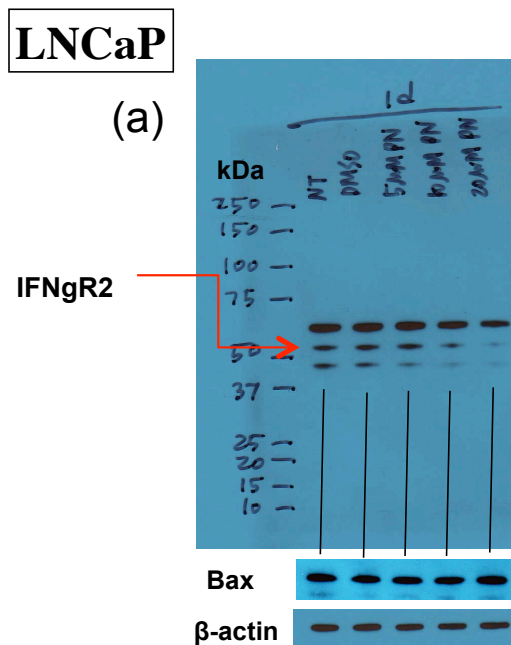
C4-2B cells were treated by lentivirus encoding shRNA targeting IFN γ R2 or no-targeting shRNA. Three days after the infection of lentivirus, cell culture images were taken, and the images are shown above. Cells treated by IFN γ R2 targeting shRNA showed significant increase of spontaneous cell death. Left bottom image of Western blot result shows the confirmation of the decrease of IFN γ R2 by shRNA targeting IFN γ R2. The decrease of IFN γ R2 levels by shRNA was not dramatic as expected from the increased spontaneous cell death, however, it may be because we were able to analyze only survived cells in shRNA-treated cells (cells with dramatic decrease of IFN γ R2 might have dead and disappeared). Please see more detail in Method section.

Fig.11 IFN γ R2 shRNA suppressed tumor growth and promoted cisplatin effects
(Each line represents each mouse treated with cancer cell and drug (or PBS))



One million cells were injected to the subcutaneous tissue of nude mouse, and the growth of these cells were monitored by measuring the size of tumor formed under the skin of these mice. Before injection to the mice, cells were treated by shRNA targeting IFN γ R2 (IFN γ R2 sh-RNA) or no targeting shRNA. Mice were treated by cisplatin every week as described in Method section.

Fig.12 Parthenolide treatment decreased IFN γ R2 expression without changes in Bax expression



LNCaP cells were cultured (1 million cells/10 ml/10 cm dish) for 24 hrs in the presence of Parthenolide at various concentration (5, 10, and 20 μ M). Parthenolide was added as DMSO solution at the final DMSO concentration of 0.1%. DMSO (0.1%) only was used as a control (DMSO). NT: no treatment, i.e. no DMSO and no Parthenolide. Expression levels of IFN γ R2, Bax, and beta actin was analyzed by Western blot using 10 μ g of total protein cell lysates in each lane. Please see detail in Method section.